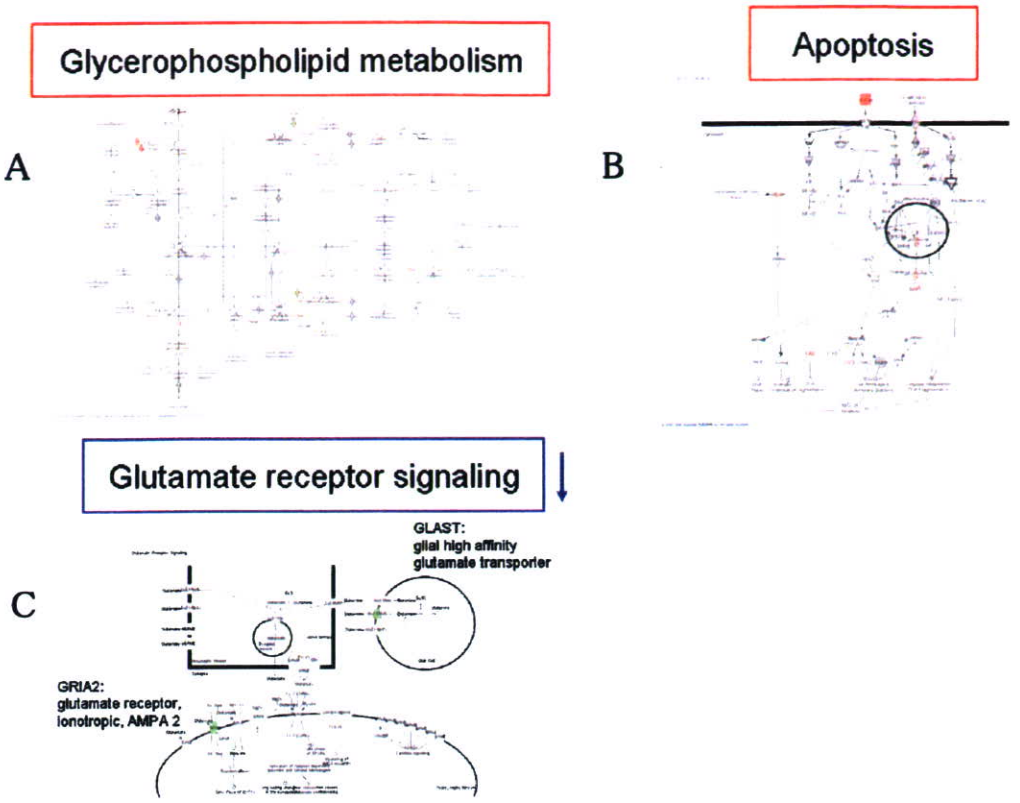

Fig4　10 週齢投与 24 時間後の変化

A) Glycerophospholipid metabolism に大脳で変化が認められた。他の脂質代謝の変化も認められている。 B) Apoptosis に大脳で変化が認められた。 C) Glutamate receptor signaling に海馬で変化が認められた。

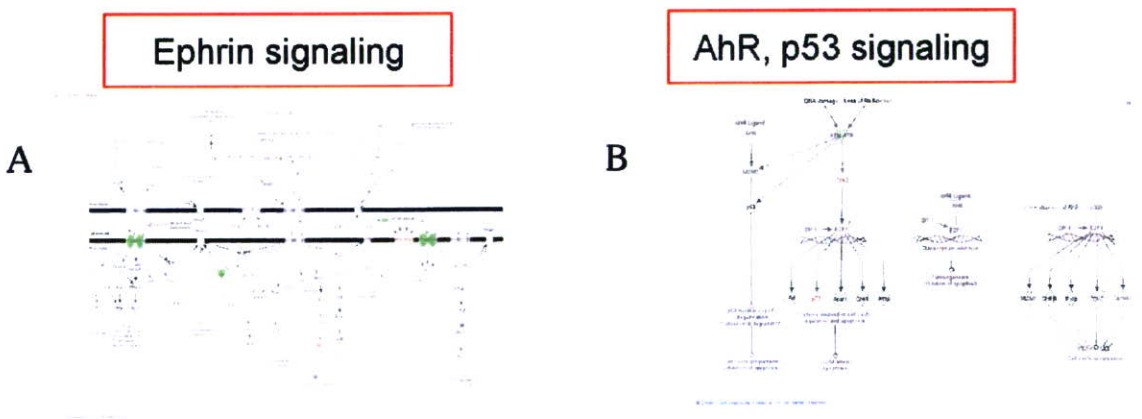

Fig5　10 週齢投与 11 週齢時の変化

A) Ephrin signaling に大脳で変化が認められた。 B) AhR, p53 signaling に海馬で変化が認められた。

厚生労働科学研究費補助金(化学物質リスク研究事業)
分担研究報告書

細胞を用いた*in vitro* における化学物質・薬物の影響解析

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研究要旨

バルプロ酸やカルバマゼピン等の抗けいれん剤の生殖発生毒性機構を解明するためにマウス胚性幹細胞(ES 細胞)ならびにマウス神経芽腫由来 N1E-115 細胞を用いて解析した。その結果、カルバマゼピンやバルプロ酸は、マウス ES 細胞の外胚葉系への分化を促進し、バルプロ酸は N1E-115 細胞では Gadd45a 遺伝子や NF2 遺伝子の発現調節を介して神経細胞の分化を促進することが明らかとなった。

A. 研究目的

バルプロ酸やカルバマゼピン等の抗けいれん剤は、神経管欠損などの生殖発生毒性を有することが知られているが、その詳細な毒性機構は明らかになっていない。本研究では、マウス ES 細胞や神経芽腫由来 N1E-115 細胞を用いて神経発生に及ぼす抗けいれん剤の影響を行う。

B. 研究方法

1. ES 細胞を用いたカルバマゼピンの細胞毒性解析

EST 法は、ES 細胞が特別な誘導を行うことなく自然に心筋細胞に分化するという特性を利用した評価方法である。ES 細胞を培養する際、薬物を加えることにより、心筋細胞への分化障害を指標としている。薬物による ES 細胞分化抑制効果は、マウス ES 細胞株を用いて行う。解析を行う化学物質・薬物を添加した試験液に ES 細胞を調整し、ディッシュの蓋の内面に 700 細胞/20 μl の懸濁滴を。蓋を裏返してディッシュに被せ、湿潤状態で 37℃,3 日間懸滴培養する。その後、液滴内に形成された胚様体 (EBs)をさらに浮遊培養して EBs をさらに成熟させる。2 日後、24 穴マルチプレートに EBs を各ウエルに1個ずつ移し、各試験液で静置培養する。5 日間経過後に倒立位相差顕微鏡にて各ウエル毎の心筋細胞の鼓動の有無を調べる。ES 細胞の分化度は、細胞の鼓動を認めるウエル数の割合をすべてのウエル数の百分率から算出し、各濃度段階のウエル数の百分率から ID_{50} を求めている。さらに、ES 細胞と 3T3 細胞の薬物による細胞毒性数値 IC_{50} 求め、ID_{50} とともにモデル計算式に挿入して、薬物の毒性の指標とした。さらに、カルバマゼピンを添加した ES 細胞を用いて未分化細胞、内胚葉、中胚葉、外胚葉組織に特異的な遺伝子マーカーの発現解析を行い、カルバマゼピンの細胞分化に及ぼす影響を観察した。

2. N1E-115 細胞を用いたバルプロ酸の細胞毒性解析

神経生理の分野においては神経細胞の軸索や樹状突起の長さ、数、分岐数など形態の異常性を観察する手法は一般的である。N1E-115 細胞に血清を添加すると細胞の分化は起こらないが、血清を除去すると神経突起の伸長など細胞が分化することが知られている。血清を添加し細胞の分化を抑制した状態の N1E-115 細胞にバルプロ酸を添加すると神経突起の伸長がみられる。この薬物による神経細胞分化への影響について解析を行い、バルプロ酸による神経突起伸長の分子生物学的解析を行う。

(倫理面への配慮)

直接ヒト検体の解析を行う場合には研究機関での倫理委員会に申請を行い承認が得られた後研究を行う。動物実験に関しては動物愛護法を遵守し、研究施設の動物実験指針に従い、実験動物の使用、飼養および保管の改善にも最大限努力する。

C. 研究成果

1. マウス胚性幹細胞(ES 細胞)を用いたカルバマゼピン(CBZ)の毒性評価ならびにその機序

EST 法にてカルバマゼピンの毒性評価を行った。カルバマゼピンの IC_{50} は NIH3T3 細胞で、0.24mM、ES 細胞で 0.31mM、ID_{50} は、28.24 μg/ml であり、毒性は弱毒性と評価された(表1)。ES 細胞の分化におけるカルバマゼピンの影響を観察するために薬物添加後10日目のES細胞よりRNA を抽出し、

表1 VPAとCBZの毒性比較

	VPA	CBZ
有効血中濃度	0.40 – 0.70 mM	0.02 – 0.05 mM
IC50-ES	2.58 mM	0.31 mM
IC50-3T3	2.25 mM	0.24 mM
ID50-ES	1210.81 μg/ml	28.24 μg/ml
Class(毒性度)	II (morderate)	II (morderate)
未分化マーカー	濃度依存的増加	濃度依存的増加
内胚葉マーカー	GATA6, TTR, AFP、ALBの濃度依存的減少	初期分化マーカーGATA6の濃度依存的上昇、後期マーカーTTR, HNF1, ALBの濃度依存的減少
中胚葉マーカー	BMP4、Nkx2.5、MLC-2v、ANF の濃度依存的減少	初期分化マーカーBMP4、Nkx2.5(心筋)の濃度依存的上昇、後期分化マーカーMLC-2v、ANFの濃度依存的減少
外胚葉マーカー	ニューロンのマーカーSynaptophysin、NFHの濃度依存的上昇、グリア細胞のマーカーGFAP、Olig2、DM20の先駆性し	神経の初期マーカーNestin、ニューロンのマーカーSynaptophysinの濃度依存的上昇(VPAに比して高濃度に少ない)、グリア細胞のマーカーGFAP、Olig2、DM20の濃度依存的上昇
考察	治療域内濃度： ES細胞の内胚葉、中胚葉系への分化を抑制し、外胚葉系の神経に誘導して(ニューロン)異的な分化誘導を促進	治療域内濃度 ES細胞の各細胞への分化誘導にほとんど影響無し。 高濃度域： 内胚葉、中胚葉のごく初期の誘導に対しては促進、中～後期発生には、抑制傾向。外胚葉系の神経に関しては、分化促進傾向であるが、VPAより弱い。
奇形発現率 (Ishida et al. Acta Paediatr 2004)	Total 35/268 (13.1%) Severe 25/268 (9.7%)	Total 46/703 (6.5%) Severe 28/703 (4.0%)

内胚葉、中胚葉、外胚葉特異的マーカーの遺伝子発現量をRT-PCR 法にて解析した。その結果、カルバマゼピン添加により ES 細胞は、高濃度領域において内胚葉、中胚葉の初期の誘導に対しては、促進傾向がみられ、中興期発生に対しては抑制傾向がみられた。外胚葉系の神経細胞マーカーでは分化促進傾向がみられたが、その作用はバルプロ酸よりも軽度であった(表1)。

2. N1E-115 細胞をバルプロ酸の細胞毒性解析

N1E-115 細胞はマウス神経芽腫由来細胞で培養液に血清を含んだ条件で培養を行うと未分化な細胞の状態で増殖するが、培養液から血清を除去すると神経突起の伸長がみられ、細胞分化が進行することが知られている。血清を含む培養液にて N1E-115 細胞を培養し、その細胞にバルプロ酸を添加したところ神経突起の伸長がみられた。バルプロ酸による N1E-115 細胞の神経突起伸長効果に関与する分子生物学的メカニズムを解明するためにジーンチップを用いて変動する遺伝子の解析を行った。その結果、NF2 遺伝子の発現(merlin:NF2 遺伝子産物)と Paxillin が亢進することが明らかとなった(図1)。

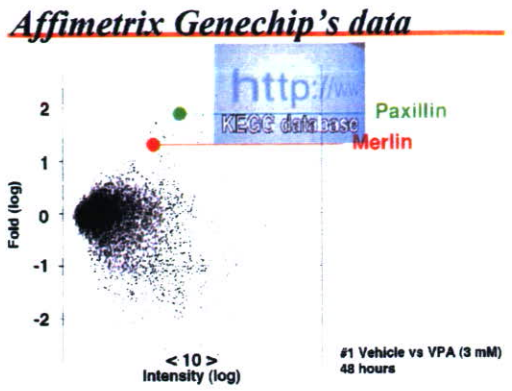

図1　ジーンチップによる遺伝子解析

RT−PCRおよびウエスタンブロット法により merlin の発現について解析したところVPA添加後 N1E−115 細胞の神経突起に一致して発現が増加しているのが確認された(図2)

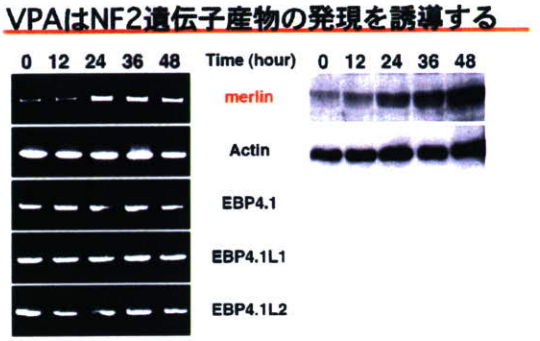

図2　N1E−115 細胞におけるバルプロ酸の神経突起伸長作用のメカニズム

次にVPAによる N1E−115 細胞の神経突起作用における Paxillin と merlin の相互作用について解析を行った。Paxillin と merlin の両方を共発現させた細胞では神経突起の伸展作用が認められた(図3)。

N1E−115 細胞において Paxillin と merlin を共に発現させると、神経突起が伸びてくることより、バルプロ酸は、Paxillin と merlin の遺伝子発現を亢進させ、神経突起作用を有しているものと考えられた(図4)。

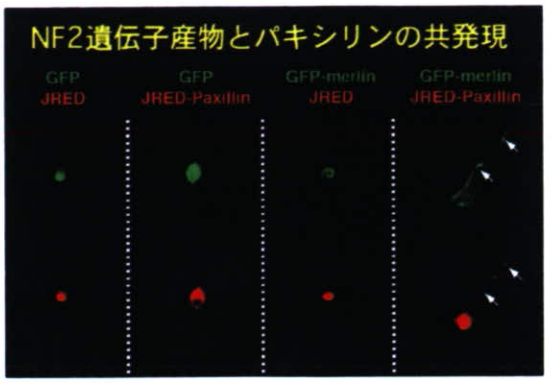

図3　Paxillin と merlin の共発現細胞における神経突起の伸展作用

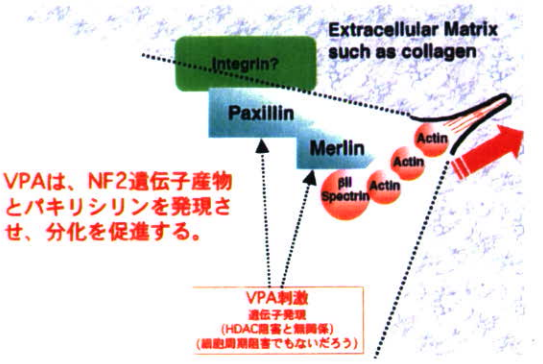

図4　N1E−115 細胞におけるバルプロ酸の NF2 を介する神経突起伸長作用のメカニズム

D. 考察

1. カルバマゼピンおよびバルプロ酸による胎児への影響は、神経管欠損などの生殖発生毒性があることが知られている。今回マウス ES 細胞を用いた試験では、ともに弱毒性の評価であったが、マウス ES 細胞でのバルプロ酸の IC_{50} が治療濃度域内(0.3−0.7mM)の 0.56mMであり、また神経系の発生により強く影響を及ぼしていることなどよりカルバマゼピンよりもバルプロ酸の方が奇形発症率が高いものと推定された。

2. N1E−115 細胞における神経突起作用に及ぼすバルプロ酸の作用は、NF2 遺伝子等を介していることが明らかとなった。バルプロ酸の神経細胞の分化に影響を及ぼすことは個体レベルで報告

されており、今回のN1E-115細胞での結果はこれを裏付けるものと考えられる。

E. 結論

マウスES細胞及びN1E-115細胞でバルプロ酸の細胞毒性効果ならびに細胞分化に及ぼす影響について分子生物学的手法により明らかにした。今後、この結果は個体レベルでのバルプロ酸の催奇形性の発症のメカニズムの解明に有用であると考えられる。

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G. 知的財産所有権の出願・登録状況(予定も含む)

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

研究成果の刊行に関する一覧表

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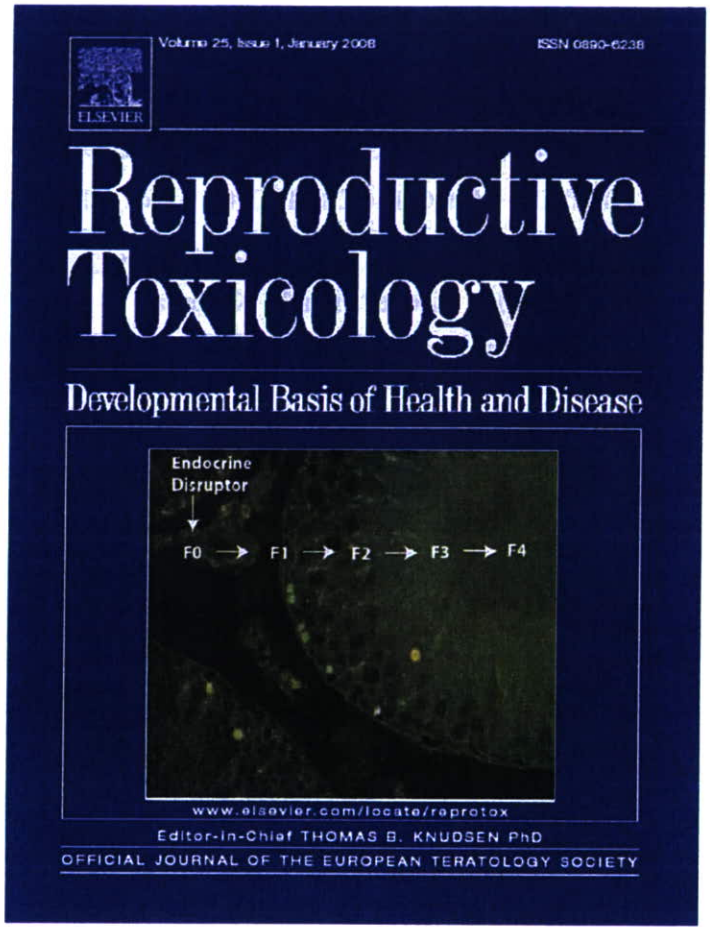

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Two-generation reproductive toxicity study of the rubber accelerator *N,N*-dicyclohexyl-2-benzothiazolesulfenamide in rats

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Abstract

Male and female Crl:CD(SD) rats were fed a diet containing rubber accelerator *N,N*-dicyclohexyl-2-benzothiazolesulfenamide (DCBS) at 0, 80, 600 or 4500 ppm throughout the study beginning at the onset of a 10-week pre-mating period and continuing through the mating, gestation, and lactation periods for two generations. At 4500 ppm, decreases in the body weight, body weight gain, and food consumption were found in F0 males and females. No changes in the estrous cyclicity, copulation index, fertility index, gestation index, delivery index, number of implantations, precoital interval, or gestation length were observed in any generation at any dose of DCBS. Delayed preputial separation at 4500 ppm as well as delayed vaginal opening and higher body weight at the age of vaginal opening at 600 and 4500 ppm were found in the F1 generation. A transient change in performance in a water-filled multiple T-maze was found at 600 and 4500 ppm in F1 females. There were no compound-related changes in number of pups delivered, sex ratio of pups, viability of pups, anogenital distance, surface righting reflex, negative geotaxis reflex, mid-air righting reflex, pinna unfolding, incisor eruption, or eye opening in the F1 and F2 generations. The body weight of F1 and F2 male and female pups was lowered at 4500 ppm. Reduced uterine weight of the weanlings was noted in the F1 generation at 4500 ppm and in the F2 generation at 600 and 4500 ppm. The data indicate that the NOAEL of DCBS for two-generation reproductive toxicity is 80 ppm (5.2 mg/kg bw per day) in rats.

Keywords: *N,N*-Dicyclohexyl-2-benzothiazolesulfenamide; Rubber accelerator; Two-generation reproductive toxicity; Developmental toxicity; Rat

1. Introduction

N,N-Dicyclohexyl-2-benzothiazolesulfenamide (DCBS) is a sulfenamide accelerator. The sulfenamide accelerator class of rubber accelerators has been manufactured in the USA for over 60 years [1]. Sulfenamide accelerator compounds are widely used in the manufacture of automotive compartments and industrial rubber products such as tires, hoses, conveyer belts, bushings seals, gaskets and windshield wiper blades, and the typical usage for sulfenamide accelerators is from 0.5 to 4 parts accelerator per every 100 parts of rubber [1]. Sulfenamide accelerator materials are shipped extensively throughout the world from manufacturing plants located in North America, South America, Europe, Asia and Africa [1]. DCBS was produced in Japan with an annual production level of about 1000 tonnes in 1990–1993 and 1900 tons in 2000–2003, and most of this amount was sold and handled domestically [2]. DCBS is used as an accelerator of vulcanization and is completely reacted in the vulcanizing process [2]. DCBS is regulated for use in articles in contact with food in Germany, but this compound is not regulated for use in FDA food contact applications [3]. Exposure of workers handling sulfenamide accelerator materials is likely to be highest in the area of materials packaging. During material packout at the manufacturing site and to a lesser degree during weigh-up activities at the consumer site, there is potential for skin and inhalation exposure. Although consumer exposure would be minimal, the most likely route of consumer exposure is skin contact from rubber or latex articles [1].

Only up to 6% biodegradation for DCBS was determined in a ready biodegradability test, and a measured log K_{ow} value of 4.8 suggests that DCBS may have a high bioaccumulation potential [2]. The possibility of such a chemical compound entering

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into biological systems has aroused great concern regarding its toxicological potential. Generally, biological effects produced by chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity are relevant to humans [4]. However, very little information on the toxicity of DCBS has been published. Vorobera (1969) [5] reported that the oral LD50 value was 8500 mg/kg bw in male mice and that repeated inhalation exposure of male rats for 15 days, daily, 2 h/day, at 350–400 mg/m^3 caused mucous membrane irritation. Although the toxic effects of DCBS have been briefly summarized by the European Chemical Bureau [6] and EPA [1], descriptions regarding the toxicity of DCBS are insufficient to assess the adverse effects of this compound. The EPA [1] noted that the oral LD50 values were 1077–10000 mg/kg bw in rats, the oral NOAEL for 44-day repeated dose toxicity was higher than 100 mg/kg bw per day in rats, and no effects on reproduction were observed at doses up to 400 mg/kg bw per day in rats. Toxicity studies including acute toxicity, in vitro genotoxicity, and repeat dose toxicity combined with reproductive/developmental toxicity studies of DCBS were performed as a part of the Safety Examination of Existing Chemical Substances and Chemical Safety Programmes by the Japanese Government [7]. These toxicity studies are summarized in the IUCLID Data Sets [8], OECD Screening Information Data Sets [2] and the Hazard Assessment Sheet [9]. We previously reported results of repeat dose toxicity combined with a reproductive/developmental toxicity screening test of DCBS showing that DCBS at 400 mg/kg bw per day possessed a deleterious effect on reproduction and development and caused a marked decrease in the number of live pups as well as a total loss of pups until postnatal day (PND) 4 [10]. The primary effects may be on the gestation index for dams and live birth index for pups, which both appear to be affected at multiple points along the female reproductive process; the viability of neonatal pups may also be affected. The previous study was performed in compliance with the OECD guideline for a Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test [11,12], but this screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. In order to further evaluate the reproductive and developmental toxicity of DCBS in rats, a two-generation reproductive toxicity study was conducted. We examined reproductive and developmental endpoints such as sexual development, estrous cyclicity, anogenital distance (AGD), physical and functional development, serum hormone levels, and sperm count and motility.

2. Materials and methods

This study was performed in 2006–2007 at the Safety Research Institute for Chemical Compounds Co. Ltd. (Sapporo, Japan) in compliance with OECD guideline 416 Two-generation Reproduction Toxicity Study [13] and in accordance with the principles for Good Laboratory Practice [14], "Law for the Humane Treatment and Management of Animals" [Law No. 105, 1 October 1973, revised 22 December 1999, Revised Law No. 221; revised 22 June 2005, Revised Law No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, 28 April 2006] and "Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, 1 June 2006].

2.1. Chemical and dosing

N,N-Dicyclohexyl-2-benzothiazolesulfenamide (DCBS, CAS No. 4979-32-2) was obtained from Ouchishinko Chemical Industrial Co. Ltd. (Tokyo, Japan). DCBS in the form of off white to tan granules is very slightly soluble in water and methanol but soluble in oil, and its melting point is 100–105 °C, density at 21 °C is 1230 kg/m^3, and molecular weight is 347 [3]. The DCBS (Lot no. 508001) used in this study was 99.7% pure, and it was kept in a sealed container under cool (1–8 °C) and dark conditions. The purity and stability of the chemical were verified by analysis using high-performance liquid chromatography before and after the study. Rats were given dietary DCBS at a concentration of 0 (control), 80, 600 or 4500 ppm. The dosage levels were determined based on the results of our previous dose-finding study in male and female rats fed a diet containing DCBS at 0, 1500, 3000, 6000 or 10,000 ppm (0, 83, 172, 343 or 551 mg/kg bw per day in males and 0, 126, 264, 476 or 707 mg/kg bw per day in females) for a total of eight weeks beginning 16 days before mating in males and a total of nine weeks in females throughout the mating, gestation and lactation periods beginning 16 days before mating. In that study, we found reduced body weight gain in males at 6000 ppm and higher and females at 3000 ppm and higher, reduced number of implantations at 6000 ppm and higher, decreased absolute and relative weight of the spleen in females at 6000 ppm and higher, reduced number of pups born at 10000 ppm, lowered body weight of pups at 6000 ppm and higher, and decreased absolute and relative weight of the spleen in male weanlings at 1500 ppm and higher and female weanlings at 3000 ppm and higher [15]. Dosed diet preparations were formulated by mixing DCBS into an appropriate amount of a powdered basal diet (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) for each dietary concentration. The control rats were fed a basal diet only. Analysis showed that DCBS was homogeneous in the diet and stable for at least 21 days in a room temperature, and formulations were maintained in a room temperature for no more than 21 days. Generally, diet was replaced every 1 week.

2.2. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male and female rats at 4 weeks of age were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for eight days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. One hundred and ninety two rats were randomly assigned 24/sex/group and all animals were assigned a unique number and ear tattooed prior to the start of the experiment. Animals were housed individually in suspended aluminium/stainless steel cages except during the acclimation, mating and nursing periods. From day 17 of pregnancy to the day of weaning, individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc.).

Animals were reared on a basal diet or diet containing DCBS and filtered tap water *ad libitum* and maintained in an air-conditioned room at 22 ± 3 °C, with a humidity of 50 ± 20%, a 12-h light (8:00–20:00)/dark (20:00–8:00) cycle, and ventilation at 10–15 times/h.

2.3. Experimental design

Twenty-four rats (5-week-old males and females)/sex/group were fed a diet containing DCBS at 0, 80, 600 or 4500 ppm for 10 weeks prior to the mating period. Each female F0 rat was mated with a male rat of the same dosage group, with administration of DCBS in the diet continuing throughout the mating period. Administration of DCBS was continued throughout gestation and lactation. Twenty-four male and 24 female F1 weanlings (1 male and 1 female

in each litter) in each group were selected as F1 parents on PNDs 21–25 to equalize the body weights among groups. The day on which F1 parental animals were selected was designated as 0 week of dosing for the F1 generation. The administration of DCBS in the diet was not suspended during PNDs 21–25. F1-selected rats were administered DCBS in the diet with the respective formulation for 10 weeks prior to the mating period and mated as described above. Administration of DCBS in the diet was continued throughout the mating, gestation, and lactation periods. On PND 26, F1 weanlings not selected for breeding and all F2 weanlings were necropsied.

2.4. Mating procedures

Each female was mated with a single male of the same dosage group until copulation occurred or the mating period had elapsed. The mating periods for F0 and F1 animals were three weeks. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered evidence for successful mating. The day of successful mating was designated as day 0 of pregnancy. F1 females that did not mate during the 3-week mating period were cohabited with other males from the same group who had been proven to copulate. For F1 matings, cohabitation of siblings was avoided.

2.5. Parental data

All adult rats were observed twice a day for clinical signs of toxicity, and body weights and food consumption were recorded weekly. For females exhibiting evidence of successful mating, body weight and food consumption were recorded on days 0, 7, 14, and 20 of pregnancy and days 0, 4, 7, 14, and 21 of lactation. Daily vaginal lavage samples of each F0 and F1 female were evaluated for estrous cyclicity throughout the 2-week pre-cohabitation period and during cohabitation until evidence of copulation was detected. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles. After weaning of their pups, parental female rats were necropsied at the proestrous stage of the estrous cycle. For each female, the number of uterine implantation sites was recorded.

2.6. Litter data

Once insemination was confirmed, female rats were checked at least three times daily at days 21–25 of pregnancy to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21 (the day of weaning). The day on which parturition was completed by 13:00 was designated as PND 0. Total litter size and the numbers of live and dead pups were recorded, and live pups were counted, sexed, examined grossly, and individually weighed on PNDs 0, 4, 7, 14, and 21. On PND 4, litters were randomly adjusted to eight pups comprising of four males and four females. No adjustment was made for litters of fewer than eight pups. Selected pups were assigned a unique number and limb tattooed on PND 4.

2.7. Developmental landmarks

All F1 and F2 pups were observed daily for pinna unfolding on PNDs 1–4, incisor eruption beginning on PND 8, and eye opening beginning on PND 12. One male and one female F1 and F2 pup selected from each dam was evaluated for the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18 [16]. All F1 offspring were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. Body weight of the respective F1 rats was recorded on the day of preputial separation or vaginal opening. The AGD was measured using calipers on PND 4 in all F1 and F2 pups, and the AGD per cube root of body weight ratio was calculated [17].

2.8. Behavioral tests

Spontaneous locomotor activity was measured with a multi-channel activity monitoring system (Supermex; Muromachi Kikai Co. Ltd., Tokyo, Japan) in 10 male and 10 female F1 rats selected from each group at 4 weeks of age. Rats were placed individually in transparent polycarbonate cages ($27.6W \times 44.5D \times 20.4H$ cm, CL-0108-1, Clea Japan Inc., Tokyo, Japan) under an infrared sensor that detects thermal radiation from animals. Spontaneous motor activity was determined for 10 min intervals and for a total of 60 min.

A test in a water-filled multiple T-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The apparatus was similar to that described by Biel [18]. The water temperature of the maze was kept 22–23 °C. As a preliminary swimming ability test, each rat was allowed to swim three times in a straight channel on the day before the maze trial, and then tested in the maze with three trials per day for the next consecutive three days. The elapsed time between entry into the water at the starting point and touching the goal ramp as well as the number of errors were recorded. To prevent exhaustion of the rats, no animal was allowed to remain in the water for more than 3 min in any trial.

2.9. Termination/necropsy-adults

Parental rats were necropsied: males after the parturition of paired female, and females after weaning of their pups. Ages on the day of the scheduled terminal sacrifice were 19–20 weeks old in F0 males, 21–22 weeks old in F0 females, 18 weeks old in F1 males and 19–20 weeks old in F1 females. The proestrous stage of the estrous cycle was characterized by examination of the vaginal smears of female rats on the day of necropsy. A complete necropsy was performed on all rats found dead and those killed at the scheduled terminal sacrifice. Live rats were euthanized by exsanguination under ether anesthesia. The external surfaces of the rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. Weights of the brain, pituitary, thyroid, thymus, liver, kidney, spleen, adrenal, testis, epididymis, seminal vesicle (with coagulating glands and their fluids), ventral prostate, uterus and ovary were recorded. Weights of the thyroid and seminal vesicle were measured after fixation. Major organs were stored in 10% neutral buffered formalin. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol.

Histopathological evaluations in F0 and F1 adults were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin: the liver, pituitary, thymus, thyroid, kidney, spleen, adrenal, bone marrow, mesenteric lymph node, Peyer's patches, testis, epididymis, seminal vesicle, coagulating gland, ventral prostate, ovary, uterus, vagina and mammary gland of all males and females in the control and highest dose (4500 ppm) groups and of females with abnormal estrous cycles, of males and females without evidence of copulation or insemination and of females with abnormal delivery or totally dead pups in all groups. Any organs or tissues of F0 and F1 adults showing gross alterations were evaluated histopathologically.

In ten each F1 females of the control and highest dose groups, the primordial follicles were counted [19]. The right ovary was fixed in 10% neutral buffered formalin and then dehydrated and embedded in paraffin in a longitudinal orientation by routine procedures. Sections were cut serially at 5 μm and every 20th one was serially mounted on slides and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.10. Termination/necropsy-pups

Following adjustment of litter size on PND 4, culled pups were euthanized by inhalation of carbon dioxide and subjected to a gross external and internal necropsy. No tissues from these pups were collected.

The weanlings not selected to become parents were euthanized and necropsied as described for the adults. Organ weights of one male and one female F1 and F2 weanling selected from each dam was measured as described above for adults. The weights of the pituitary and thyroid were not determined in weanlings. All pups found dead before weaning were also necropsied.

In all male and female F1 and F2 weanlings whose organs were collected, histopathological evaluations of the thymus, liver and spleen in the control and 4500 ppm groups were performed after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin.

2.11. Hematological and blood biochemical parameters

On the day of the scheduled terminal sacrifice, blood samples were collected from the abdominal aorta of adult rats under ether anesthesia.

Hematological examinations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Blood samples were analyzed for the following hematological parameters, using 2K-EDTA as an anticoagulant: white blood cell count (WBC) and differential leukocyte count.

Blood biochemical evaluations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Serum samples obtained from centrifuged whole blood were analyzed for biochemistry parameters such as total protein, albumin and globulin.

2.12. Serum hormone levels

On the day of the scheduled terminal sacrifice, blood samples were collected from the abdominal aorta of adult rats. Eight males and eight proestrous females of the F0 and F1 generations from each group were selected randomly for blood collection. Hormone levels were determined by Panapharm Laboratories Co. Ltd. (Uto, Japan). Serum levels of testosterone, 5α-dihydrotestosterone (DHT), luteinizing hormone (LH), and follicle stimulating hormone (FSH) in males, and estradiol, progesterone, LH, and FSH in females were measured. The testosterone, DHT, estradiol, and progesterone concentrations were measured using a double antibody kit (Diagnostic Products Corp., Los Angeles, CA or Diagnostic Systems Laboratories Inc., Webster, TX). Serum concentrations of LH and FSH were measured using (rat LH)[125I] and (rat FSH) [125I] assay systems (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), respectively.

2.13. Sperm parameters

Sperm parameters were determined for all F0 and F1 male adults, except dead males, on the day of the scheduled terminal sacrifice. The right testis was used to count testicular homogenization-resistant spermatid heads. The right cauda epididymis was weighed and used for sperm analysis. Sperm motility was analyzed using a computer-assisted cell motion analyzer (TOX IVOS, Hamilton Thorne Biosciences, Beverly, MA). The percentage of motile sperm and progressively motile sperm as well as their swimming speed and pattern were determined. After the recording of sperm motion, the cauda epididymal fluid was diluted and sperm were enumerated using a hemacytometer under a light microscope. A sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. The sperm were stained with eosin and mounted on a slide glass. Two hundred sperm in each sample were examined under a light microscope, and the percentage of morphologically abnormal sperm was calculated.

2.14. Statistical analysis

Statistical analysis of offspring before weaning was carried out using the litter as the experimental unit.

Body weight, body weight gain, food consumption, length of estrous cycle, precoital interval, gestation length, numbers of implantations and pups delivered, delivery index, sperm parameters, hematological and blood chemical parameters, hormone levels, organ weight, organ/body weight ratio (relative organ weight), reflex response time, age displayed pinna unfolding, incisor eruption, and eye opening, age and body weight at sexual maturation, parameters of behavioral tests, AGD, AGD/cube root of body weight ratio, and the viability of pups were analyzed for statistical significance in the following way. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances. If the variances were equivalent, the groups were compared by one-way analysis of variance (ANOVA). If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalent variances, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pairwise comparisons were made by Mann–Whitney *U*-test. The incidence of pups with changes in clinical and gross internal observations, and reflex completion rate of pups were analyzed by Wilcoxon rank sum test. The number of primordial follicles in the control and highest dose groups was analyzed in the following way. Variance ratio was analyzed by *F*-test. Since the variance ratio was equivalent, the groups were compared by Student's *t*-test. The incidence of females with normal estrous cycles, copulation index, fertility index, gestation index, neonatal sex ratio, and completion rate of the reflex response were analyzed by Fisher's exact test.

The 0.05 level of probability was used as the criterion for significance.

3. Results

3.1. Clinical observations, body weight and food consumption during the pre-mating, mating, gestation, and lactation periods (F0 and F1)

There were no compound-related clinical signs of toxicity in either male or female F0 and F1 rats during the pre-mating, mating, gestation, or lactation periods. One F0 male at 80 ppm was euthanized in 11 weeks of dosing because of a moribund condition resulting from accidental injury in the home cage. One F1 female without any apparent clinical signs of toxicity died on day 5 of lactation in the control group, and no abnormal necropsy findings were found.

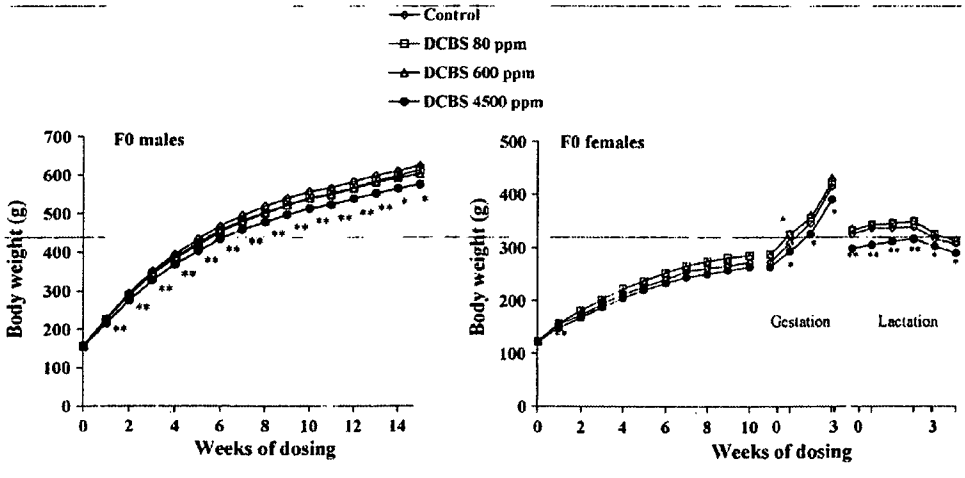

Fig. 1. Body weight of F0 males and females. *Significantly different from the control, $p < 0.05$. **Significantly different from the control, $p < 0.01$.

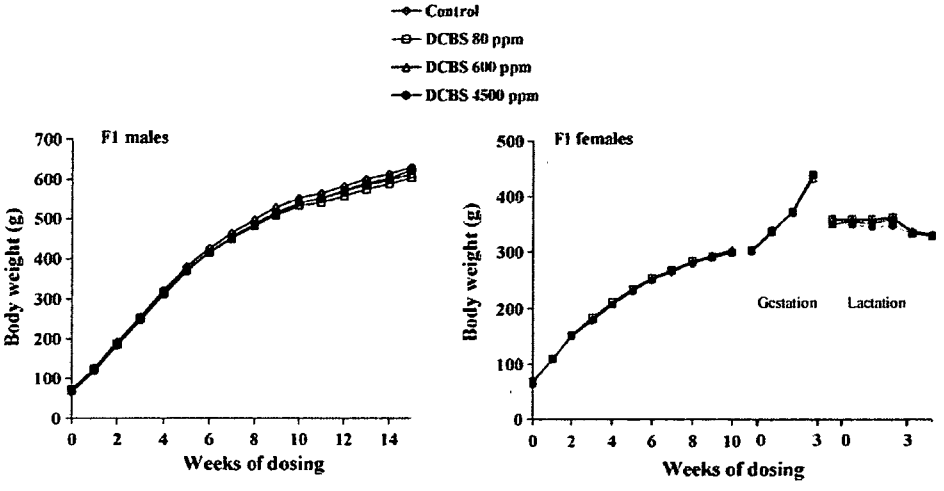

Fig. 2. Body weight of F1 males and females.

The body weights of F0 males and females during dosing are shown in Fig. 1. The body weight and body weight gain of male F0 rats were significantly lowered throughout the dosing period at 4500 ppm. At this dose, the body weight and body weight gain of F0 females were significantly reduced during the first week of dosing and throughout pregnancy and lactation. No compound-related changes in the body weight or body weight gain were noted in F0 males and females at 80 and 600 ppm.

Fig. 2 shows the body weights of F1 males and females during the dosing period. The body weight and body weight gain of F1 males and females exhibited no significant differences between the control and DCBS-treated groups.

There was a significant decrease in food consumption during weeks 1–8 and 13–14 of dosing in F0 males and during the first week of dosing and days 14–21 of lactation in F0 females at 4500 ppm. No significant changes in food consumption were observed in F0 rats of both sexes at 80 and 600 ppm (data not shown).

In F1 male rats, a significant decrease in food consumption was found during weeks 4–7 of dosing at 80 ppm, during week 6 of dosing at 600 ppm and during week 4 of dosing at 4500 ppm. No significant changes were observed in food consumption in F1 females at any dose (data not shown).

The mean daily intakes of DCBS were 5.2, 39 and 291 mg/kg bw in F0 males, 7.2, 54 and 416 mg/kg bw in F0 females, 5.9, 44 and 331 mg/kg bw in F1 males, and 7.4, 55 and 417 mg/kg bw in F1 females for 80, 600 and 4500 ppm, respectively.

3.2. Estrous cyclicity (F0 and F1 females)

Table 1 presents the estrous cyclicity of F0 and F1 females. All F0 females showed normal estrous cycles in all groups, and the length of the estrous cycles was not different between the control and DCBS-treated groups. Although one F1 female each in the control and 600 ppm groups displayed extended diestrous vaginal smears, no significant changes in the incidence of females having normal estrous cycles or length of the estrous cycles were observed.

3.3. Reproductive effects (F0 parents/F1 offspring and F1 parents/F2 offspring)

The reproductive and developmental parameters for F0 parent/F1 offspring are presented in Table 2 . In F0 parent animals, all pairs in all groups copulated, although two females in the con-

Table 1
Estrous cyclicity of F0 and F1 females

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 females				
No. of females examined	24	24	24	24
Females with normal estrous cycles (%)[b]	100	100	100	100
Length of estrous cycles (days)	4.05 ± 0.16[a]	4.01 ± 0.06	4.04 ± 0.15	4.01 ± 0.06
F1 females				
No. of females examined	24	24	24	24
Females with normal estrous cycles (%)[b]	95.8	100	95.8	100
Length of estrous cycles (days)	4.21 ± 0.34	4.05 ± 0.21	4.25 ± 1.08	4.07 ± 0.24

[a] Values are given as the mean ± S.D.
[b] Incidence of females with normal estrous cycles (%) = (no. of females with normal estrous cycles/no. of females examined) × 100.

Table 2
Reproductive and developmental data for F0 parents/F1 offspring and F1 parents/F2 offspring

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 parents/F1 offspring				
No. of pairs	24	24	24	24
Copulation index (%)[b]				
Male/female	100/100	100/100	100/100	100/100
Fertility index (%)[c]	91.7	100	100	100
No. of pregnant females	22	24	24	24
Precoital interval (days)	2.4 ± 1.2[a]	2.8 ± 1.1	2.4 ± 1.0	2.4 ± 1.1
Gestation index (%)[d]	100	100	100	100
Gestation length (days)	22.1 ± 0.4	22.2 ± 0.4	22.0 ± 0.3	22.1 ± 0.3
No. of implantations	13.5 ± 2.1	13.9 ± 1.4	14.6 ± 1.3	13.2 ± 1.5
Delivery index (%)[e]	94.9	94.9	94.3	94.8
No. of pups delivered	12.8 ± 2.1	13.2 ± 1.6	13.8 ± 1.5	12.5 ± 1.7
No. of litters	22	24	24	24
Sex ratio of F1 pups[f]	0.528	0.554	0.506	0.525
Viability index during lactation (%)[g,h,i]				
Day 0	99.0	99.3	99.7	99.0
Day 4	98.7	98.2	96.6	97.6
Day 21	100	99.0	99.5	99.5
Male pup weight during lactation (g)				
Day 0	6.9 ± 0.5	6.7 ± 0.6	6.7 ± 0.6	6.6 ± 0.7
Day 4	11.2 ± 1.1	10.5 ± 1.2	10.5 ± 1.4	10.3 ± 1.0[e]
Day 7	18.6 ± 1.8	18.1 ± 1.7	17.7 ± 2.5	16.7 ± 1.6[e]
Day 14	37.2 ± 3.6	36.8 ± 2.4	36.0 ± 4.0	33.6 ± 2.5[e,*]
Day 21	62.3 ± 5.6	62.2 ± 3.7	60.2 ± 6.3	55.3 ± 4.8[e,*]
Female pup weight during lactation (g)				
Day 0	6.5 ± 0.5	6.3 ± 0.5	6.3 ± 0.5	6.3 ± 0.6
Day 4	10.9 ± 1.3	10.1 ± 1.4	10.0 ± 1.2	9.9 ± 1.0[e]
Day 7	18.1 ± 1.9	17.1 ± 2.3	17.2 ± 2.3	16.2 ± 1.4[e]
Day 14	36.3 ± 3.5	34.8 ± 3.6	35.0 ± 4.0	32.8 ± 2.6[e]
Day 21	60.7 ± 5.2	58.5 ± 6.0	58.2 ± 6.5	53.7 ± 4.5[e,**]
F1 parents/F2 offspring				
No. of pairs	24	24	24	24
Copulation index (%)[b]				
Male/female	100/100	100/100	91.7/100	100/100
Fertility index (%)[c]	95.8	91.7	91.7	100
No. of pregnant females	23	22	22	24
Precoital interval (days)	2.7 ± 1.0	2.6 ± 1.4	2.6 ± 1.2	2.8 ± 1.7
Gestation index (%)[d]	100	100	95.5	100
Gestation length (days)	22.3 ± 0.4	22.2 ± 0.4	22.1 ± 0.4	22.1 ± 0.3
No. of implantations	14.1 ± 3.2	13.5 ± 3.7	13.0 ± 4.2	14.3 ± 2.1
Delivery index (%)[e]	90.4	92.9	88.9	91.3
No. of pups delivered	12.7 ± 3.6	12.6 ± 3.7	12.0 ± 4.2	13.0 ± 2.4
No. of litters	23	22	21	24
Sex ratio of F2 pups[f]	0.488	0.516	0.557	0.522
Viability index during lactation (%)[g,h,i]				
Day 0	98.7	99.7	98.3	95.9
Day 4	95.9	94.2	93.1	88.4
Day 21	100[j]	100	97.0	97.7[j]
Male pup weight during lactation (g)				
Day 0	6.8 ± 0.9	6.7 ± 0.8	6.7 ± 0.5	6.7 ± 0.6
Day 4	11.0 ± 2.3	11.1 ± 2.6	10.0 ± 2.1	10.0 ± 1.4[j]
Day 7	18.5 ± 2.7[j]	18.4 ± 3.8	17.1 ± 2.8	15.9 ± 2.3[j,*]
Day 14	37.1 ± 4.0[j]	37.8 ± 6.3	35.5 ± 3.8	32.3 ± 4.1[j,**]
Day 21	62.5 ± 7.0[j]	63.4 ± 9.4	60.6 ± 5.6	53.5 ± 5.9[j,**]

Table 2 (*Continued*)

	DCBS (ppm)			
	0 (control)	80	600	4500
Female pup weight during lactation (g)				
Day 0	6.5 ± 1.0	6.3 ± 0.7	6.3 ± 0.4[k]	6.3 ± 0.7
Day 4	10.5 ± 2.3	10.5 ± 2.5	9.7 ± 2.0[k]	9.5 ± 1.5[l]
Day 7	17.6 ± 2.9[j]	17.7 ± 3.8	16.3 ± 2.8[k]	15.5 ± 2.2[l]
Day 14	35.9 ± 4.1[j]	36.6 ± 5.7	33.5 ± 4.9[k]	31.7 ± 3.9[l,*]
Day 21	59.6 ± 6.6[j]	60.7 ± 8.5	56.3 ± 7.0[k]	52.0 ± 5.7[l,**]

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[c] Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[f] Sex ratio = total no. of male pups/total no. of pups.
[g] Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.
[i] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 4 after cull) × 100.
[j] Data were obtained from 22 litters because one female that died on day 5 of lactation was excluded from the data.
[k] Data were obtained from 20 litters because one female had no female pups.
[l] Data were obtained from 23 litters because one female that experienced a total litter loss on day 3 of lactation was excluded from the data.
[*] Significantly different from the control, $p < 0.05$.
[**] Significantly different from the control, $p < 0.01$.

trol group did not become pregnant, and all pregnant females in all groups delivered live pups. There were no significant differences in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F1 pups delivered, sex ratio of F1 pups, or viability of F1 pups during lactation between the control and DCBS-treated groups. No malformed F1 pups were found in any groups. A significantly lower body weight was observed in male and female F1 pups at 4500 ppm on PNDs 4, 7, 14 and 21.

The reproductive and developmental parameters for F1 parent/F2 offspring are also shown in Table 2. Two F1 males in the 600 ppm group did not copulate. One female in the control group and two females each in the 80 and 600 ppm groups did not become pregnant. One pregnant female in the 600 ppm group did not deliver. One dam in the control group died on day 5 of lactation, and her pups were euthanized. One dam experienced a total litter loss by PND 3 at 4500 ppm. No significant changes in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F2 pups delivered, sex ratio of F2 pups, or viability of F2 pups during lactation were observed. Oligodactyly in one female of the control group and microphthalmia in one male at 80 ppm were observed. Body weights of F2 pups at 4500 ppm were significantly lowered on PNDs 7, 14 and 21 in males and PNDs 14 and 21 in females.

3.4. Developmental landmarks (F1 and F2)

Physical development of F1 and F2 pups is presented in Table 3. There was no significant difference in the age of male and female F1 and F2 pups that displayed pinna unfolding, or eye opening between the control and DCBS-treated groups. The completion of incisor eruption was delayed in male and female F1 pups at 80 ppm and in male and female F2 pups at 80 and 4500 ppm. The AGD and AGD per cube root of body weight ratio in male and female F1 and F2 pups were not significantly different between the control and DCBS-treated groups.

Reflex ontogeny in F1 and F2 pups is shown in Table 4. All male and female F1 pups in all groups completed the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18. In F1 pups, no significant difference was observed in the response time of the surface righting reflex or the negative geotaxis reflex between the control and DCBS-treated groups. Of the F2 pups, one female did not complete the surface righting reflex and one male did not complete the mid-air righting reflex at 80 ppm, one female did not complete the mid-air righting reflex at 600 ppm, and one female did not complete the negative geotaxis reflex at 4500 ppm; however, no significant difference was found between the control and DCBS-treated groups in the completion ratio and response time for these reflexes.

Table 5 presents data on sexual development in F1 rats. Although a significant delay in the age of preputial separation in males was noted at 4500 ppm, the body weight at the age of preputial separation was not significantly different between the control and DCBS-treated groups. In females, a significantly delayed age of vaginal opening and a higher body weight at the age of vaginal opening were found at 600 and 4500 ppm.

3.5. Behavioral effects (F1)

Spontaneous locomotor activity in 10 min intervals for a total of 60 min was not significantly different between the control and DCBS-treated groups in male and female F1 rats (data not shown).

Fig. 3 shows the results of the water filled T-maze test in F1 males and females. The pre-test swimming trials in the straight channel on the first day of the T-maze test revealed that all F1

Table 3
Physical development in F1 and F2 pups

	DCBS (ppm)			
	0 (control)	80	600	4500
F1 pups				
No. of litters examined	22	23	24	24
Age at pinna unfolding (days)				
Male	2.7 ± 0.5[a]	2.7 ± 0.5	2.9 ± 0.3	2.7 ± 0.5
Female	2.6 ± 0.6	2.6 ± 0.6	2.9 ± 0.4	2.7 ± 0.5
Age at incisor eruption (days)				
Male	10.2 ± 0.6	10.8 ± 0.6**	10.3 ± 0.6	10.5 ± 0.4
Female	10.1 ± 0.6	10.7 ± 0.7**	10.2 ± 0.7	10.2 ± 0.6
Age at eye opening (days)				
Male	14.5 ± 0.6	14.5 ± 0.5	14.7 ± 0.5	14.6 ± 0.5
Female	14.4 ± 0.6	14.5 ± 0.7	14.4 ± 0.4	14.5 ± 0.5
AGD				
Male pup AGD (mm)	5.60 ± 0.28	5.50 ± 0.28	5.51 ± 0.41	5.54 ± 0.28
Male pup AGD/($BW^{1/3}$)	2.51 ± 0.09	2.52 ± 0.08	2.52 ± 0.12	2.55 ± 0.09
Female pup AGD (mm)	3.02 ± 0.11	2.95 ± 0.14	2.99 ± 0.14	2.96 ± 0.14
Female pup AGD/($BW^{1/3}$)	1.36 ± 0.05	1.37 ± 0.06	1.39 ± 0.04	1.38 ± 0.04
F2 pups				
No. of litters examined	23	22	21	23
Age at pinna unfolding (days)				
Male	2.7 ± 0.8	2.7 ± 0.7	2.8 ± 0.6	2.7 ± 0.5
Female	2.7 ± 0.8	2.7 ± 0.8	2.8 ± 0.4[c]	2.6 ± 0.6
Age at incisor eruption (days)				
Male	9.7 ± 0.7[b]	10.6 ± 0.9**	9.9 ± 0.6	10.3 ± 0.8*
Female	9.8 ± 0.7[b]	10.4 ± 0.8*	10.0 ± 0.6[c]	10.4 ± 0.9*
Age at eye opening (days)				
Male	14.4 ± 0.7[b]	14.6 ± 0.8	14.3 ± 0.7	14.6 ± 0.6
Female	14.3 ± 0.6[b]	14.4 ± 0.8	14.4 ± 0.5[c]	14.5 ± 0.7
AGD				
Male pup AGD (mm)	5.54 ± 0.51	5.60 ± 0.55	5.39 ± 0.56	5.47 ± 0.38
Male pup AGD/($BW^{1/3}$)	2.50 ± 0.12	2.53 ± 0.14	2.51 ± 0.12	2.55 ± 0.08
Female pup AGD (mm)	2.93 ± 0.19	2.91 ± 0.22	2.88 ± 0.19[c]	2.85 ± 0.18
Female pup AGD/($BW^{1/3}$)	1.34 ± 0.04	1.34 ± 0.06	1.35 ± 0.03[c]	1.35 ± 0.05

[a] Values are given as the mean ± S.D.
[b] Data were obtained from 22 litters because one dam that died on day 5 of lactation was excluded from the data.
[c] Data were obtained from 20 litters because one female had no female pups.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

rats in each group could swim satisfactorily, and no significant changes in the elapsed time to traverse the straight channel were observed. In males, no significant differences were observed between the control and DCBS-treated groups in the elapsed time and number of errors in on days 2–4 of the T-maze test. In females, a significantly longer elapsed time at 600 and 4500 ppm and more errors at 4500 ppm were noted on day 2 of the T-maze test. There were no significant differences in the elapsed time or number of errors on days 3 and 4 of the T-maze test in female rats between the control and DCBS-treated groups.

3.6. Necropsy and histopathology (F0, F1 and F2)

There were no compound-related gross lesions or microscopic alterations in the reproductive organs of F0 and F1 males and females showing reproductive difficulties. No compound-related gross lesions or remarkable microscopic alterations of tissues and organs, including the reproductive organs, were noted in F0 and F1 males and females in the highest dose group and dead animals before the scheduled terminal sacrifice. In the histopathological examinations of the ovary in F1 females, no significant difference was noted in the number of primordial follicles (mean ± S.D.) between the control (323 ± 57) and 4500 ppm (255 ± 109) groups. There were no compound-related gross lesions or microscopic alterations in male and female F1 and F2 pups, including pups that died before weaning (data not shown).

3.7. Organ weights (F0 adults)

The body weight at the scheduled terminal sacrifice was significantly lowered at 4500 ppm in males and females. Sig-

Table 4
Reflex ontogeny in F1 and F2 pups

	DCBS (ppm)			
	0 (control)	80	600	4500
F1 pups				
No. of pups examined (male/female)	22/22	24/24	24/24	24/24
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Surface righting reflex response time (s)				
Male	2.1 ± 1.6[a]	1.5 ± 0.5	2.4 ± 2.3	1.8 ± 1.2
Female	2.8 ± 3.4	1.6 ± 0.6	1.9 ± 0.9	3.4 ± 3.9
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Negative geotaxis reflex response time (s)				
Male	14.5 ± 8.0	15.4 ± 8.2	13.8 ± 6.4	16.0 ± 7.5
Female	15.3 ± 6.8	14.1 ± 6.0	15.4 ± 6.2	18.3 ± 7.6
Mid-air righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
F2 pups				
No. of pups examined (male/female)	22/22	22/22	21/20	23/23
Surface righting reflex completion rate (%)				
Male/female	100/100	100/95.5	100/100	100/100
Surface righting reflex response time (s)				
Male	2.5 ± 1.6	2.2 ± 1.8	1.7 ± 0.5	2.1 ± 1.9
Female	2.6 ± 1.8	2.4 ± 2.0[b]	2.5 ± 1.7	3.2 ± 4.5
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/95.7
Negative geotaxis reflex response time (s)				
Male	15.3 ± 6.3	17.2 ± 7.4	14.4 ± 5.7	16.1 ± 4.9
Female	16.9 ± 7.2	14.0 ± 6.5	12.6 ± 8.1	16.0 ± 6.2[c]
Mid-air righting reflex completion rate (%)				
Male/female	100/100	95.5/100	100/95.0	100/100

Surface righting reflex on postnatal day 5, negative geotaxis reflex on postnatal day 8 and mid-air righting reflex on postnatal day 18 were examined three times.
Completion rate (%) = (number of animals showing all successful responses of thee trials/number of animals examined) × 100.
[a] Values are given as the mean ± S.D.
[b] Data were obtained from 21 pups.
[c] Data were obtained from 22 pups.

Table 5
Sexual development in F1 males and females

	DCBS (ppm)			
	0 (control)	80	600	4500
Male preputial separation				
No. of males examined	24	24	24	24
Age (days)	41.3 ± 1.6[a]	41.4 ± 1.6	41.8 ± 1.6	42.8 ± 1.5**
Body weight (g)	226.9 ± 20.3	226.5 ± 18.5	228.3 ± 17.0	229.6 ± 17.5
Female vaginal opening				
No. of females examined	24	24	24	24
Age (days)	29.6 ± 1.0	30.0 ± 1.7	31.2 ± 1.7**	31.1 ± 1.3**
Body weight (g)	104.6 ± 9.4	109.1 ± 10.6	112.1 ± 13.8*	112.3 ± 9.1*

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

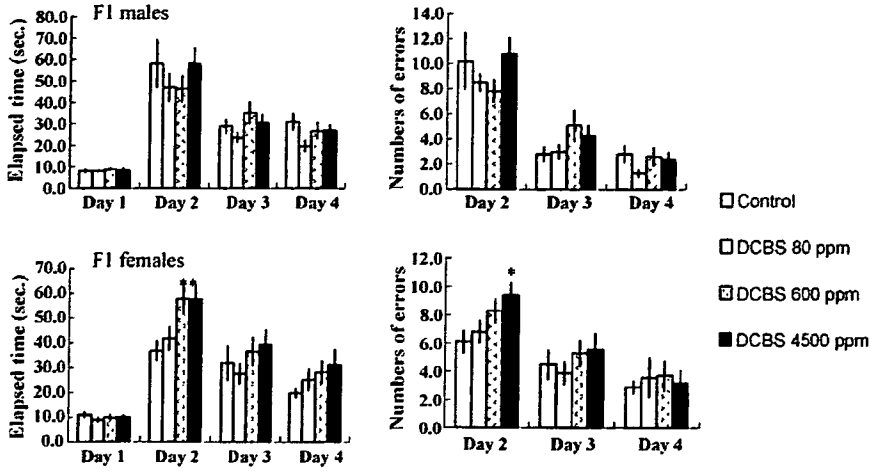

Fig. 3. Performance in water-filled multiple T-maze in F1 males and females. Each rat was allowed to swim in a straight channel on day 1, and then tested in the maze for the next consecutive three days (days 2–4). Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

nificantly decreased absolute weights of the spleen and adrenal gland, and increased relative weights of the brain, thyroid, liver, kidney and testis were detected at 4500 ppm in males. A significant increase in the absolute weights of the brain at 80 and 600 ppm and the pituitary at 80 ppm, and decrease in the relative weight of the spleen at 80 and 600 ppm was observed in F0 females. Significantly decreased absolute weight of the spleen, and increased relative weights of the brain, kidney, and adrenal gland were found at 4500 ppm in females (data not shown).

3.8. Organ weights (F1 weanlings and adults)

The organ weights of male and female F1 weanlings are presented in Table 6. The body weight at the scheduled sacrifice was significantly lowered in males and females at 4500 ppm. The relative weights of the kidney at 80 ppm and the liver at 600 ppm were significantly higher in males. Significant decreases in the absolute weights of the brain, thymus, liver, kidney, adrenal gland, epididymis, and ventral prostate, and decrease in both the absolute and relative weights of the spleen, and increase in the relative weights of the brain, liver and testis were all observed at 4500 ppm in males. A significantly increased relative weight of the kidney at 80 ppm and decreased absolute weight of the ovary at 600 ppm was found in females. The absolute weights of the brain, thymus, liver, kidney, spleen, adrenal, ovary and uterus, and the relative weight of the spleen were significantly lowered at 4500 ppm in females. In this group, significantly higher relative weights of the brain and liver were also observed in females.

Table 7 shows the organ weights of male F1 adults at the scheduled terminal sacrifice. The absolute and relative weights of the thymus were significantly lower at 80 ppm in males. A significantly decreased absolute weight of the brain, decreased absolute and relative weights of the seminal vesicle, increased relative weight of the kidney, and increased absolute and relative weights of the liver were noted at 4500 ppm in males.

The organ weights of female F1 adults at the scheduled terminal sacrifice are shown in Table 8. The absolute weight of the brain at 80 and 600 ppm, and the relative weights of the liver and kidney, and the absolute and relative weights of the adrenal gland at 4500 ppm were significantly increased.

3.9. Organ weights (F2 weanlings)

Table 9 presents the organ weights of male F2 weanlings. The body weight at sacrifice was significantly reduced at 4500 ppm. A significant decrease in the absolute and relative weight of the spleen was observed at 80 ppm. The relative weights of the liver and kidney were significantly higher at 600 ppm. At 4500 ppm, a significantly decreased absolute weight of the adrenal gland, decreased absolute and relative weights of the thymus and spleen, and increased relative weights of the brain, liver, and kidney were noted in males.

Table 9 also presents the organ weights of female F2 weanlings. A significant decrease in the body weight at sacrifice was found at 4500 ppm. The relative weight of the thymus was significantly lower at 80 ppm. Significantly increased relative weights of the liver and kidney, and reduced absolute and relative weights of the uterus were found at 600 ppm. At 4500 ppm, significantly decreased absolute weights of the brain and spleen, and absolute and relative weights of the thymus and uterus, and increased relative weights of the brain, liver and kidney were noted in females.

3.10. Hematological and blood biochemical parameters (F0 and F1 adults)

A significantly higher percent of lymphocytes was observed in male F0 adults at 4500 ppm and in female F1 adults at 600 ppm. In female F0 and male F1 adults, no significant difference was noted in the WBC or differential leukocyte count between the control and DCBS-treated groups. There were no significant changes in biochemistry parameters such as total protein, albumin and globulin in male and female F0 and F1 adult rats (data not shown).